A gain of function mutation causing skeletal overgrowth in the *rapunzel* mutant

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**Introduction**

The molecular mechanisms underpinning growth and the establishment of proper size and form are largely unknown. Vertebrate morphology is inextricably related to the growth and form of the supporting skeletal structures. Identifying mutations that result in defects in bone morphology might provide insights regarding the molecular mechanisms that regulate bone shape and size.

We used the zebrafish fin and axial skeleton as a model to examine genes critical for vertebrate bone development. Fin rays are composed of multiple segments, each segment in turn comprised of two hemirays (lepidotrichia) of dermal bone in apposition, surrounding an intra-ray mesenchyme (Santamaria *et al*., 1992). In addition to bone, the mesenchymal compartment contains nerves, blood vessels, pigment cells and undifferentiated fibroblasts, all surrounded by a basement membrane and an overlying epithelium. Lepidotrichia are covered on both surfaces by a monolayer of bone-forming osteoblasts (scleroblasts) that form as undifferentiated mesenchymal cells in the distal portion of the intra-ray proliferate (Goldsmith *et al*., 2003), condense laterally along actinotrichia, differentiate, and begin secreting bone matrix (Goss, 1978; Haas, 1962). Fin growth occurs via the sequential, distal addition of new segments of bone to each fin ray (Goss and Stagg, 1957; Haas, 1962) with fin length ultimately being determined by the number and size of these individual fin ray segments (Iovine and Johnson, 2000). Zebrafish mutants have been described that affect both the number of segments (rapunzel and long fin) (Goldsmith *et al*., 2003; Iovine and Johnson, 2000) and the size of individual segments (short fin) (Iovine and Johnson, 2000) and some molecular details of these events have been elucidated. For example, segment length is regulated in part by the function of the gap junction protein, connexin 43 (cx43) and hypomorphic alleles of the zebrafish cx43 gene result in the zebrafish short fin mutant (Hoptak-Solga *et al*., 2007; Iovine *et al*., 2005). To date, the molecular identity of mutations affecting segment number have not been published, nor have mutations been described that affect growth of both the fin ray (dermal) and the axial skeleton.

We have cloned and characterized the zebrafish mutant rapunzel. Adult zebrafish display a heterozygous overgrowth phenotype of both the fin ray and the axial skeleton. In addition, rapunzel has a distinct, homozygous lethal embryonic phenotype (Goldsmith *et al*., 2003) and the axial skeleton. In addition, rapunzel has a distinct, homozygous lethal embryonic phenotype (Goldsmith *et al*., 2003) and Fig 1). Utilizing this embryonic phenotype, we identified a missense mutation in a previously undescribed gene (*rpz*) of unknown function. Sequence analysis demonstrated that this mutation is not a common polymorphism and morpholino knockdown of *rpz* completely suppressed the *rapunzel* embryonic phenotype, demonstrating that *rapunzel* is a gain of function allele. In addition, extensive analysis of the *rapunzel* critical region demonstrates that *rpz* founds a family of five related genes. Finally, the region of the predicted rapunzel protein containing the missense mutation is highly conserved in teleosts. Therefore *rapunzel* provides an opportunity to gain new information about the fundamental mechanisms shaping vertebrate morphology.

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**Abstract**

Mechanisms that regulate the growth and form of the vertebrate skeleton are largely unknown. The zebrafish mutant *rapunzel* has heterozygous defects in bone development, resulting in skeletal overgrowth, thus identification of the genetic lesion underlying *rapunzel* might provide insight into the molecular basis of skeletogenesis. In this report, we demonstrate that the *rapunzel* mutant results from a missense mutation in the previously uncharacterized *rpz* gene. This conclusion is supported by genetic mapping, identification of a missense mutation in *rapunzel* in a highly conserved region of the *rpz* gene, and suppression of the *rapunzel* homozygous embryonic phenotype with morpholino knockdown of *rpz*. In addition, *rpz* transcripts are identified in regions correlating with the homozygous embryonic phenotype (head, pectoral fin buds, somites and fin fold). This report provides the first gene identification for a mutation affecting segment number in the zebrafish fin and development of both the fin ray (dermal) and the axial skeleton.

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Materials and methods

Fish husbandry

Wild type fish stocks used for these studies were from the C32 (Rawls et al., 2003) and AB strains. Fish were reared at a constant temperature of 25 °C and maintained on a 14L: 10D photoperiod. Fish were fed three times daily with both micropellets (Hikari, Aquatic Eco-Systems) and brine shrimp (Biomarine, Aquafauna Biomarine).

Alizarin red and Alcian blue staining

Zebrafish were euthanized in Tricaine and fixed in 4% buffered paraformaldehyde. Embryos were then stained for developing cartilage with Alcian blue as described by Schilling et al., 1996. Embryos and larvae were stained with alizarin red as described by Walker and Kimmel, 2007. Following staining, embryos were stored in glycerol. Whole-mount specimens were imaged using a Nikon SMZ1500 stereomicroscope and photographed using Nikon ACT-1 software. Images were processed in Photoshop CS3 (Adobe).

Calcein staining

A 0.2% calcein solution was made by dissolving 2 g of calcein in 1 L of MilliQ water and adjusting the pH to ~7.0 with NaOH. Fish were immersed in calcein solution for 5–10 min and then washed with fresh system water for 10 min to clear away excess dye as previously described (Du et al., 2001). Fish were then anesthetized in 0.03% Tricaine for viewing and imaging as described above.

Sirius red staining

Wild type and rapunzel heterozygous fish (18 months of age) were euthanized in Tricaine, their viscera removed, and fixed over night in 4% buffered paraformaldehyde at 4 °C. Following fixation the fish were decalcified in 0.35 M EDTA, pH 7.8 for several days. Fish were then embedded in paraffin and 5 μM sections were cut on a microtome. Sections were then stained with Sirius red as previously described (Blumer et al., 2004).

MicroCT and DEXA scanning

rapunzel heterozygotes (2 months, 8 months, and 18 months) and their wild type siblings were euthanized in Tricaine and fresh frozen at −20 °C until scanned. Prior to scanning the fish were embedded in 1% agarose. Fish were scanned on a μCT40, Scanco Medical (5 kV, 177 A, 200 ms, 16 μm voxel size). Manufacturer’s software (Eval v6.0) was used to calculate bone volume and apparent mineral density (calibrated to the manufacturer’s hydroxyapatite [HA] mineral phantom). For DEXA scanning, total body bone mineral density (BMD) was measured by DEXA using a PIxImus scanner GE/Lunar; Madison, WI). Calibration was performed daily with a standard phantom as suggested by the manufacturer. The precision of whole-body BMD, assessed by the root mean square method is 1.34% (coefficient of variation).

Microscopy

General microscopy was performed with a Nikon SMZ1500 stereomicroscope and images captured using Nikon DXM1200F digital camera and Nikon ACT-1 software. Fluorescent images were taken using an Olympus MVX10 microscope and Olympus MicroSuite.
software. Nomarski images were captured using an Olympus IX71 microscope and MicroSuite Biological Suite software. All images were processed using Photoshop CS2 (Adobe).

Mapping

We localized the *rapunzel* mutation to linkage group (LG) 16 by centromere linkage analysis (Johnson et al., 1996) using simple sequence repeat (SSR) markers (Shimoda et al., 1999). SSR markers were then used to finely map the *rapunzel* mutation (780 meioses with zero recombinations). In addition to mapping with SSR markers, we also developed single nucleotide polymorphism (SNP) markers from genes localized to this region (Hukriede et al., 2001). A description of these markers and their respective polymorphisms may be found in Supplemental Table 1.

**Fig. 2.** Cloning of *rapunzel*. (A) A meiotic map representing approximately 47 kb of the *rapunzel* region on chromosome 16. The critical region contains three paralogous genes (*rpz*, *rpz2* and *rpz3*). Two additional paralogues (*rpz4* and *rpz5*) are also present on chromosome 16, outside of the critical region. All five genes are located on the same strand. Marker positions are shown along with their number of crossovers from *rapunzel* (see Supplemental data for more details regarding marker descriptions). (B) Electropherograms showing the T269A mutation in heterozygous and homozygous *rapunzel* embryos. W indicates the single nucleotide change of thymine to adenine present on chromosomes carrying the *rapunzel* allele at position 269. The translated amino acid sequence for wild type and *rpz* homozygotes is shown below each electropherogram. The T269A mutation results in an amino acid change from valine to glutamic acid at amino acid 90 in the encoded protein. (C) A dendogram demonstrating that the five *rapunzel*-like paralogues subdivide into two clades.

In situ hybridizationTemplates for sense and anti-sense probes were cloned by RT-PCR using forward (ACACAATGGCGACCAATGAAT) or reverse (TAGAGGAAAAAGGCAATAGAGGC) primers targeted against *rpz*. PCR amplifiers were gel-purified (Qiagen) and TA-cloned (pCRII, Invitrogen). Insert-containing plasmids were grown and harvested (Maxi-prep,
Table 1

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<th>Embryos</th>
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<th>Total Embryos</th>
<th>rpz/rpz phenotype</th>
<th># confirmed rpz/rpz</th>
<th>Fraction confirmed rpz/rpz</th>
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Adult rapunzel heterozygotes were intercrossed and the resulting embryos injected at the 1-cell stage with a morpholino targeting rpz (MOrpz) (1:2 dilution in 1× Danieau buffer). For controls, we injected either a morpholino targeting rpz2 (MOrpz2) (1:2 dilution in 1× Danieau buffer) or a 5 base pair mismatched morpholino against rpz (MOrpz_5bm) (1:5 dilution in 1× Danieau buffer). At 72 hpf, individual embryos were injected with MOrpz and collected for genomic DNA extraction. The rpz/rpz genotype indicates the number of embryos resembling the morpholino's presence. For each embryo, the region flanking the rpz/24 mutation (T629A) was PCR amplified using the genomic DNA of each injected embryo as template. PCR amplicons were purified (Invitrogen), digested (DdeI) and analyzed by gel electrophoresis. The restriction digest pattern identifies the genotype of each embryo (+/+, rpz/+, rpz/rpz). The fraction of confirmed rpz/rpz indicates the number of embryos with the rpz/rpz genotype. The fraction of confirmed rpz/rpz embryos equals the # confirmed rpz/rpz embryos divided by the total embryos.

Fig. 3. Morpholino knockdown of rpz suppresses the homozygous rapunzel phenotype. Heterozygous rapunzel adult zebrafish were intercrossed and the resulting embryos injected at the 1–2 cell stage with either a morpholino targeting the start site of rpz (MOrpz) (A), a morpholino targeting rpz2 (MOrpz2) (B) or a 5 base pair mismatched control morpholino (MOrpz_5bm) (C). A. Inhibition of MOrpz suppresses the homozygous embryonic rapunzel phenotype. The rapunzel homozygous phenotype was not suppressed in embryos injected with MOrpz2 (B) or MOrpz_5bm (C). B. DdeI digestion and electrophoresis confirmed that the embryos in A–C are all homozygous for the rapunzel2/4 allele (not shown).
The rapunzel phenotype is caused by a missense mutation in the novel rpz gene

We fine mapped rapunzel to a 46 KB critical region on chromosome 16 (Fig. 2A). This critical region contained three novel, paralogous genes as evidenced by mapped (http://www.ensembl.org/Danio_rerio/) EST sequences and tBLASTx pair-wise comparisons. Because none of the three paralogues in the critical region seemed a more likely candidate for rapunzel than the others, we examined the coding sequences of all three genes in wild type zebrafish and in rapunzel mutants.

We used 3’ and 5’ random amplification of cDNA ends (RACE) to clone two of the three paralogous genes in the rapunzel critical region. A large open reading frame for the third parologue was obtained by sequencing genomic DNA from rapunzel mutants and wild type zebrafish. One of the three genes, hereafter referred to as rapunzel (rpz), contained a missense mutation (T269A) in the mutant cDNA sequence (Fig. 2B). This T269A mutation in rpz would result in a non-conserved amino acid change (V90E) in the predicted protein sequence (Fig. 2B). To exclude the possibility T269A might be a common polymorphism, we partially sequenced rpz from 4 genetic strains of zebrafish: AB, WIK, C32 and SJD. rapunzel arose on an AB genetic background and neither our AB laboratory strain nor the other 3 polymorphic strains (WIK, C32, SJD) harbored a T269A polymorphism, although a synonymous polymorphism (G345A) was identified in C32 and SJD. In addition, a BLAST search was performed to identify all EST’s demonstrating rpz sequence similarity. Four EST’s showed sequence similarity with rpz coding sequence. None of these EST’s contained the T269A mutation, although a non-synonymous polymorphism was identified at a different site that results in a neutral amino acid change (H169Q). Finally, to be certain that the T269A mutation was not introduced during transcription of the cDNA RACE template, we partially sequenced rpz from genomic DNA in 20 homozygous mutant rapunzel embryos (scored by phenotype). In every case, genomic DNA from a rapunzel mutant harbored the T269A mutation (data not shown). No differences between mutant and wild type sequence were identified in the remaining two paralogues within the critical region, referred to as rapunzel 2 (rpz2) and rapunzel 3 (rpz3).

rpz founds a family of five paralogous genes in zebrafish

In addition to rpz, rpz2 and rpz3, further tBLASTx searches revealed two additional paralogues on chromosome 16 but outside of the critical region, which we have named rapunzel 4 (rpz4) and rapunzel 5 (rpz5) (Fig. 2A). Even though rpz4 and rpz5 were outside of the critical region, we sequenced open reading frames for both genes from mutant and wild type DNA to further characterize the rpz gene family and to ask whether any other paralogues contained additional mutations present in the mutant, but not the wild type allele. No sequence differences were identified. A dendrogram (Fig. 2C) reveals that the five members of the rpz family sort into two main clades.

rapunzel14 is a gain of function allele

To establish causality between the rapunzel14 genotype and phenotype, we took advantage of rapunzel’s homozygous phenotype. We first injected a morpholino oligonucleotide directed against the putative translation start site of rpz (MOrpz) into wild type embryos. Wild type embryos were injected at the 1-cell stage with 2–5 ng of MOrpz or its 5 base pair mismatched control morpholino (MOrpz_5bm). 98% of wild type embryos injected with MOrpz had a normal phenotype at 3 dpf (n=95). A normal phenotype was observed in 100% of control-injected (MOrpz_5bm) embryos (n=45). At higher concentrations, injections of both MOrpz and MOrpz_5bm had non-specific effects of similar magnitude (data not shown). These data raised the possibility that rapunzel14 is not a loss of function allele and furthermore, that rpz is not essential for early embryonic development. We therefore hypothesized that rapunzel14 is a gain of function allele. To test this hypothesis, we injected embryos derived from adult rapunzel/+ intercrosses with 2–5 ng of either MOrpz or MOrpz_5bm. Injection of MOrpz suppressed the homozygous rapunzel phenotype, with all of the embryos injected with MOrpz displaying a wild type phenotype at 3 dpf (n=132) (Table 1 and Fig. 3). To prove that MOrpz was indeed rescuing homozygous rapunzel embryos, we took advantage of the fact that the T269A mutation introduces a DdeI restriction site into the rpz coding sequence. All injected embryos were individually photographed 3 dpf followed by DNA extraction. Genotype was then established by Ddel digest (not shown). Twenty-four percent (32/132) of the embryos injected with MOrpz had the homozygous rapunzel genotype, but a normal phenotype (Table 1 and Fig. 3A). For control (MOrpz_5bm)
injected embryos, 21% (16/75) had a rapunzel phenotype (not statistically dissimilar from the 25% Mendelian prediction [Table 1 and Fig. 3C]) and of these 16 embryos, 94% (15/16) had a homozygous rapunzel genotype confirmed by Ddel digest. Thus misclassification of an abnormal appearing embryo as a rapunzel mutant was an extremely uncommon event. As an additional control, we used a morpholino to knock down rpz2 (MOrpz2) (the topology of the rapunzel critical region is such that rpz2’s two exons lie within the second intron of rpz [Fig. 2A]). 28% (n = 53) of MOrpz2 injected embryos had a rapunzel phenotype (again not significantly different from the 25% predicted by Mendelian inheritance [Table 1 and Fig. 3B]) and 100% (15/15) of the phenotypically abnormal embryos were confirmed to be rapunzel homozygotes by Ddel digest. Together, these data demonstrate that the rapunzel embryonic phenotype results from a gain of function mutation in the rpz gene.

Expression of rpz in wild type and mutant embryos

We hypothesize that the homozygous phenotype in rapunzel arises because of the critical alteration the genetic lesion (T269A) brings about in protein structure (V90E) and function. To provide further evidence that the T269A allele does not simply segregate in rapunzel with a separate lesion affecting the expression of an otherwise functionally normal protein product, we first looked at rpz expression in mutant and wild type embryos using in situ hybridization (ISH). At 24 hpf, rpz transcripts are localized in areas consistent with the embryonic phenotype including the somites, tail bud region adjacent to the fin fold, pectoral fin buds and the head (Figs. 4 A, B). We then asked whether rpz is expressed later during development, when definitive cartilaginous and bony elements of the skeleton begin to form. At 48 hpf, rpz expression is beginning to decrease, although the general distribution is unchanged. By 120 hpf, rpz transcript is almost completely absent (Fig. 4). Importantly, we could define no significant differences in rpz expression between mutant and wild type embryos by ISH (data not shown), consistent with the hypothesis that because T269A is a missense allele, transcript localization and quantity is unlikely to be affected. To further support the notion that rapunzelF14 is not a misexpression allele, we performed qPCR on mutant and wild type embryos. At 24 hpf, no significant differences in levels of rpz transcript are noted between wild type, heterozygous rapunzel and homozygous rapunzel embryos (one way ANOVA, p = 0.84) (Fig. 4C).

rpz is a highly conserved gene in teleosts

rpz encodes a predicted 227 amino acid protein. A blastp search revealed no significant homologies aside from orthologous genes in other teleosts; moreover a search of the Pfam database of protein families reveals no significant matches. Thus, rpz encodes a novel protein of unknown function. A translated blast search (tblastn) of multiple databases (nr, EST, HTGS, and WGS) did reveal rpz orthologues in other teleosts, including cyprinid species closely related to Danio rerio (e.g. Cyprinus carpio) and quite distantly related teleosts (e.g. Takifugu rubripes). Given the dramatic phenotype caused by the V90E mutation and the fact that this lesion is embryonic lethal, we hypothesized that Rpz protein homologs would be highly conserved, particularly around the site of the rapunzel mutation (V90). Consistent with this hypothesis, a partial alignment of Rpz predicted protein homologs (Fig. 5A) demonstrates that the V90 amino acid residue is invariably a valine, alanine, or leucine in other teleost species. Also, all rpz paralogs have a nonpolar amino acid at this position (not shown). To date, only the rapunzelF14 allele contains a non-conserved amino acid (glutamic acid) at the position normally occupied by V90.

Adult rapunzel heterozygotes display overgrowth of both the fin ray and the axial skeleton

rapunzelF14 is a homozygous, lethal, gain of function allele in zebrafish embryos. In adult zebrafish, rapunzelF14 has a heterozygous fin phenotype consisting of an excess number of fin ray segments (Goldsmith et al., 2003). In addition to overgrowth of the fin ray skeleton, adult rapunzelF14 heterozygotes have pronounced hyperossification of the axial skeleton (Fig. 6). We performed bone histomorphometry using Alcian blue, alizarin red, and Sirius red staining, along with microCT and bone densitometry (DEXA) to more closely define the skeletal overgrowth phenotype of rapunzel heterozygotes. Alcian blue, alizarin red and calcinein staining were

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**Fig. 5.** Conservation of the amino acid sequence of the rpz gene. (A) A translated blast search (tblastx) of the EST database revealed rpz orthologues in cyprinid species closely related to Danio rerio. (Genbank accession numbers for each species: Rutilus rutilus [EG531508], Cyprinus carpio [CP660604], Pimephales promelas [DT229800]) Other related teleosts were identified by a tblastx search in EMBL 2.x [7] (http://wwwensembl.org/Multi/tblastx) using the species of interest. Each protein sequence was then aligned in the area flanking the rpz mutation to look for homology. The boxed outlines the amino acid change in rapunzelF14. Only rapunzelF14 contains a non-neutral amino acid at this position. NetPhos 2.0 identified putative threonine and tyrosine phosphorylation sequences shown in grey and pink, respectively. Asterisks indicate the putative phosphorylation sites. (B) A schematic of the predicted Rpz protein structure. Numbers indicate the amino acid position. The arrow highlights the V90E mutation. Asterisks indicate putative phosphorylation sites predicted by NetPhos 2.0. Putative transmembrane domains are shown as boxes at positions predicted by TMpred.
initially used to ask whether hyperossification develops during early skeletal development (3–10 dpf) in rapunzel heterozygotes. In particular, we focused on the developing craniofacial skeleton for this question, as these are the first skeletal elements to form in developing zebrafish. At these early stages of skeletogenesis, no evidence of hyperossification is noted (Fig. 7). Using in vivo calcein staining, we first begin to see differences in bone morphology in rapunzel heterozygotes as compared to their wild type siblings in two-week-old larvae (Figs. 6A, B). The vertebrae are less scalloped in shape, although the intervertebral spaces are preserved. A similar phenotype is observed with Alcian blue/alizarin red-stained larvae at three weeks of age (Figs. 6C, D). Of note, we do not yet see a hyperossification phenotype in these 2–3-week-old heterozygous rapunzel larvae. A loss of scalloping in the vertebral bodies persists into adulthood (18 months), however by this time hyperossification of the axial skeleton is readily seen by Sirius red staining. Sections through the vertebral column show rapunzel heterozygotes have an increased amount of bone compared to their wild type siblings (Figs. 6E, F). We used both microCT (Figs. 6G–N) and bone densitometry (DEXA) (Fig. 6O) to define the time course over which the hyperossification develops in heterozygous rapunzel mutants. Increased bone mineral density in heterozygous rapunzel mutants compared to their wild type siblings is not evident at 2 months of age, but is easily quantifiable at 8 and 18 months of age.

Although we did not observe a skeletal phenotype in rapunzel heterozygotes at pre-larval stages, we used ISH to ask whether the expression of well-defined markers of skeletal development is altered. ISH was performed on 48–120 hpf embryos (heterozygous rapunzel and their wild type siblings) using markers of cartilage (col2a1) and bone (col10a1, osx) development. Consistent with previously published data, we saw strong expression in the pharyngeal arches, otic vesicle, endochondral disc, and notochord of col2a1 (Van et al., 2005) (Supplemental Figure). Expression in the developing bony skeleton, including the cleithrum, opercle, paraspinalid, and brachiostellar ray was seen with col10a1 and osx (Li et al., 2009) (Supplemental Figure). Importantly, at these pre-larval stages we did not detect differences in expression of any of these genes between heterozygous mutant and wild type embryos. These data are perhaps not surprising in light of that fact that a skeletal phenotype has not developed at these early stages. Therefore, we performed ISH on sections of young larvae (2–4 weeks of age) to ask whether the skeletal phenotype seen in heterozygous rapunzel mutants is linked to increased expression of skeletal genes. At two weeks of age we begin to see overexpression of col2a1 (Figs. 8A, B) in heterozygous rapunzel mutants. By four weeks of age, overexpression of col10a1 and osx are also observed (Figs. 8C–F). Finally, we find overexpression of col2a1, col10a1, and osx in the fins from 3-month-old rapunzel mutants (Figs. 8G–L). This increased expression of col2a1, col10a1, and osx that we observe in the caudal fin was further quantified by qPCR (Fig. 8M). Col2a1 and osx are significantly increased (p<0.05) in expression (2.65 ± 0.71 and 1.94 ± 0.43, respectively) in the fins from heterozygous rapunzel mutants versus wild type. Col10a1 is also increased in expression (1.78 ± 0.54), although this fold change did not achieve statistical significance (data represent mean ± SD).

In order to further characterize rpz expression at time points when a skeletal phenotype is apparent in heterozygous rapunzel mutants, ISH was performed on zebrafish larvae (2–4 weeks of age). At two weeks of age, rpz transcripts are seen diffusely in the vertebral column, muscle, and head (Figs. 8N, O). At three and four weeks of age, levels of rpz transcript decrease slightly, however the distribution remains unchanged (not shown). Interestingly, rpz does not show colocalization with the skeletal genes col2a1, col10a1, or osx.

Our data (see above) indicate that the gain of function phenotype in homozygous rapunzel embryos does not arise from misexpression or overexpression of the rpz gene (Fig. 4). Although unlikely, these data do not exclude the possibility that overgrowth of the rapunzel fin ray skeleton arises from misexpression or overexpression of the rpz gene in the fin, as is true in other fin overgrowth mutations such as long fin (Kathy Lovine and Stephen Johnson, not shown). Consistent with our data from rapunzel embryos, qPCR revealed no differences in levels of rpz transcripts between the fins of rapunzel heterozygotes and wild type zebrafish (not shown). We also observed no quantitative differences in rpz transcript in wild type fin regenerates and rapunzel/+ fin regenerates, when expression is normalized to wild type fins (data not shown).

Discussion

We report here the identification of a missense mutation in a novel gene, rpz. rapunzel+ describes both a homozygous embryonic phenotype (lethal) as well as a heterozygous adult phenotype (overgrowth of the axial and appendicular skeleton). Furthermore, we show that morpholino knockdown of rpz suppresses the homozygous embryonic phenotype, however morpholino knockdown of rpz in wild type embryos produces no demonstrable...
morphant phenotype. From these data we can derive the following conclusions. First, *rpz* is not essential for early embryonic development. It remains formally plausible that the morpholino knockdown of *rpz* translation in wild type embryos was partial, allowing for translation of sufficient protein to prevent a morphant phenotype. This seems less likely since identical doses of morpholino were sufficient to completely suppress the mutant phenotype, however definitive proof will require an antibody (that we are currently generating) that recognizes the endogenous Rpz protein. It also remains possible that the *rpz* family of genes has redundant functions, thus knockdown of a single paralogue will not yield a morphant phenotype, but knockdown of multiple genes could produce a loss of function phenotype (see below). Second, *rapunzelc14* is a gain of function allele. Although it remains possible that the heterozygous mutant phenotype could result from a dominant negative effect, this seems less likely given the absence of a morphant phenotype. RNA-targeting via anti-sense technology (including morpholinos) has emerged as a promising mechanism for rescuing null phenotypes induced by splice mutations (Wood et al., 2007). This approach has been used successfully in cell culture (Bruno et al., 2004; Du et al., 2007; Gebski et al., 2003; Suwanmanee et al., 2002) and in vivo (Alter et al., 2006; Lu et al., 2005; Madsen et al., 2008; van Deutekom et al., 2007). Our data provide the first example of rescue of a gain of function zebrafish mutant by morpholino injection. This has important ramifications for using zebrafish as a complex vertebrate model system to gain insight into human diseases, as a large number of human diseases are caused by gain of function missense mutations (Fisher et al., 2003). We hypothesize that the gain of function phenotype arises from accumulation of the mutant protein and we find no evidence that *rpz* is aberrantly expressed in the embryo. Again, more definitive proof must wait the generation of an antibody against the Rpz protein. We did attempt to phenocopy the *rapunzel* embryonic phenotype by over expressing wild type and mutant *rpz* RNA’s (data not shown). However, we found that injecting either mutant or wild type *rpz* RNA’s produced markedly abnormal embryos. This was likely due to the absence of an endogenous promoter regulating the expression of the injected RNA’s. *rpz* is a founding member of a novel gene family. We identified five zebrafish paralogues in the *rpz* family, both within and immediately adjacent to the *rapunzelc14* critical region. To date, *rpz* homologs have only been identified in teleosts. While zebrafish
appear to have five members of this novel gene family (see above), the number of paralogues varies in other species. tBLASTx comparisons (http://www.ensembl.org) identify six orthologous genes in *Orzias latipes* and *Gasterosteus aculeatus*, while Takifugu rubripes contains five. tBLASTx results show that all orthologous genes of these species localize to a single chromosome, as is the case in zebrafish. As shown above, both exons for *rpz2* are located within the second intron of *rpz*, although the complete coding sequence for both of these genes is contained within a single exon. Since we did not clone full-length cDNA’s for the *rpz* gene family from other teleosts, we do not know if these species share the same curious genomic arrangement as seen in zebrafish. The presence of multiple *rpz* orthologues in zebrafish raises the question of whether these genes have overlapping and redundant functions during development. We have performed double morpholino injections (*rpz* and *rpz2*) into wild type embryos (not shown) and find no evidence of redundancy to date. Morpholinos targeting the remaining three orthologues are currently being generated.

In addition to a homozygous embryonic phenotype, *rapunzel* has a heterozygous adult phenotype affecting both the fin ray (Goldsmith et al., 2003) and the axial (see above) skeleton. Interestingly, *rpz* gene expression appears strongest prior to the formation of definitive skeletal elements and does not show colocalization with well-defined skeletal genes, suggesting that *rpz* might act in a non-cell-autonomous manner. However, heterozygous *rapunzel* mutants demonstrate increased expression of the skeletal markers *col2a1*, *col10a1*, and *osx*, providing a plausible mechanistic link wherein the mutation results in an increased number or activity of bone forming cells, leading to skeletal overgrowth. These data are consistent with our previously published results (Goldsmith et al., 2003) demonstrating an expanded zone of cell proliferation in the fins of heterozygous *rapunzel* mutants. It has been shown previously (Goldsmith et al., 2003; lovine and Johnson, 2000) that fin growth in adult, wild type zebrafish is episodic and isometric. Furthermore, fin overgrowth mutations have been previously described (Goldsmith et al., 2003; Haffter et al., 1996; lovine and Johnson, 2000, 2002; van Eeden et al., 1996) wherein isometric and episodic control of fin growth is lost. *rapunzel* represents the first fin overgrowth mutant affecting segment number to be cloned. Zebrafish mutants (*dolphin, stocksteif*) causing craniofacial abnormalities and hyperossification have been previously described (Laue et al. 2008, Spoorendonk et al., 2008). These mutants differ from *rapunzel* in that they show fusion of the vertebrae (not seen in *rapunzel* mutants) and do not affect the fin ray skeleton. An additional mutant, *touchtone*, affecting both endochondral and intramembranous ossification (Elizondo et al., 2005) has been described, but again, abnormalities of the fin ray skeleton are not observed. Also, the zebrafish mutant *chihuahua* displays skeletal abnormalities and a shorter caudal fin, but these are phenotypes not seen in *rapunzel* mutants (Fisher et al., 2003). Therefore, *rapunzel* is the first mutant identified causing overgrowth of both the axial and the fin ray skeleton. Interestingly, *rapunzel*’s function is unknown; moreover cloning projects underway suggest that candidate genes for other fin overgrowth mutants do not play currently known roles in growth control (S. Johnson, not shown). Therefore, zebrafish genetics provides an important segue to identify previously uncharacterized growth control pathways in an adult, vertebrate organism.

We hypothesize that the *rapunzel* mutant phenotypes result from accumulation of the mutant protein (i.e. gain of function). Presumably a single *rapunzel*14 allele produces sufficient mutant protein to induce a phenotype in adult fish, however embryos require two copies of the mutant allele to demonstrate a phenotype. In addition, we find no evidence in *rapunzel* heterozygotes for aberrant expression leading to up regulation of the *rpz* gene in the fin (not shown), even though this is the mechanistic basis for overgrowth in other zebrafish mutants (S. Johnson and K. lovine, not shown). We were unable to localize *rpz* transcript in the fin by ISH (not shown), although ISH is notoriously unreliable for the identification of fin transcripts, even when these transcripts are present in high abundance such as during regeneration (Smith et al., 2008). How might the V90E mutation alter the function of the *rapunzel* protein? *In silico* analysis predicts that *rapunzel* encodes a transmembrane protein (Fig. 5B), a finding supported by preliminary, *in vitro* experiments. Additionally, computational analyses (NetPhos 2.0) (Fig. 5B) identify a putative phosphorylation site at T98 (IRKTVDRQ). Interestingly, the amino acid sequence of the mutant, but not of the wild type *rapunzel* allele predicts a putative phosphorylation site at Y94 (EDRQYHEVE). Perhaps conformational changes induced by the V90E mutation alter phosphorylation and downstream signaling of the *rapunzel* protein. There exists precedence for this model, as previous studies have shown that substituting glutamic acid for valine can activate the RAS kinase pathway, resulting in cellular proliferation (Turner et al., 2005), moreover malignant melanomas often contain a valine to glutamic acid mutation (Sharma et al., 2005; Turner et al., 2005). We are currently testing whether the *rapunzel* protein is phosphorylated *in vitro*. Future studies will elaborate the mechanism by which the mutant *rapunzel* protein results in overgrowth.

In summary, we have cloned *rapunzel* and identified a gain of function mutation in a novel gene (*rpz*) of unknown function. This conclusion is supported by three lines of evidence: the *rapunzel*14 mutation maps to the *rapunzel* gene, expression of the *rpz* gene in embryos is consistent with the embryonic phenotype, and abrogation of *rpz* gene activity via morpholino injection suppresses the homozygous *rapunzel*14 phenotype. In addition, the *rpz* gene is highly conserved in teleosts. Indeed, the V90 amino acid residue that is mutated in *rapunzel*14 is invariably a conserved, neutral amino acid in all species for which sequence data is available. We also demonstrate that *rapunzel* heterozygotes have increased expression of cartilage and bone-forming genes providing a mechanism for the skeletal hyperossification phenotype. Although the function of *rpz* remains unknown and must await further experimentation, the identification of the genetic basis for skeletal overgrowth in *rapunzel*14 provides new insight into the mechanisms underpinning vertebrate morphology and growth.

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**Appendix A. Supplementary data**


**References**
